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Full Length Article

Management of Basal Rot Disease of Onion with Dry Leaf Biomass of *Chenopodium album* as Soil Amendment

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Abstracts

Basal rot is a highly destructive disease of onion (*Allium cepa* L.) that is caused by the fungus *Fusarium oxysporum* Schlechtend: Fr. f. sp. *cepae* (Hans.) Snyder and Hansen. This study was carried out to examine the potential of *Chenopodium album* L. leaves for the control of this disease. In a pot trial, incorporation of dry leaf biomass of *C. album* at 3% (w/w) significantly reduced disease incidence and mortality by 63% and 89%, respectively. In laboratory bioassays, methanolic leaf extract was successively fractionated using four organic solvents viz. *n*-hexane, chloroform, ethyl acetate and *n*-butanol. Different concentrations (3.125, 6.25, …, 200 mg mL^{-1}) of these fractions were examined against the selected pathogen in malt extract broth. Chloroform fraction exhibited the best antifungal activity resulting in 96-100% reduction in the fungal biomass. Through thin layer chromatography (TLC), two unidentified compounds A (R_f 0.17) and B (R_f 0.44) were isolated from this fraction. Antifungal activity of these compounds was compared with a commercial fungicide Mancozeb. Minimum inhibitory concentrations (MIC) of compounds A and B were 62.5 and 250 μg mL^{-1}, respectively after 24 h as compared to 7.81 μg mL^{-1} MIC of the reference compound. The results of the present study conclude that antifungal compounds in chloroform fraction of methanolic leaf extract and soil amendment with dry leaf biomass of *C. album* can be used as alternatives to chemical fungicides for the management of basal rot disease of onion. © 2015 Friends Science Publishers

Keywords: Basal rot; *Chenopodium album; Fusarium oxysporum* f. sp. *cepae;* Natural fungicides; Onion

Introduction

Onion is an important vegetable crop all over the world, widely used as a condiment in preparation of soups, meat dishes, salads, sandwiches and is also cooked as a vegetable. The characteristic secondary metabolites of onions are sulfur containing alliins (cysteine sulfoxides), which add taste and sharpness and are criteria for the pharmaceutical quality (Bloem *et al.,* 2005). *Fusarium oxysporum* f. sp. *cepae* is a highly destructive pathogen that causes basal rot disease in onion resulting in significant yield losses of the crop in all growing areas of the world (Ozer and Koycu, 2004). The pathogen infects the basal stem plate of the onion bulb and degrades it, ultimately kills the whole plant (Cramer, 2000). The main sources of the inoculum are contaminated seeds and soil (Ozer and Koycu, 1997). Under field conditions, early disease symptoms are yellowing of leaves and tip dieback, and the whole plant may collapse with the development of the disease. If pathogen attacks the host plant late in the season, the symptoms may not appear until the onion bulbs are in storage (Ozer *et al.,* 2003).

Seed treatments with benomyl and soil fumigation with metam sodium or methyl bromide effectively control the disease (Jaworski *et al.,* 1978; Ozer and Koycu, 1998). However, nowadays the use of synthetic agro-chemicals has increased consumer's concern and the use of these chemicals is becoming more restraining because of their carcinogenic effects, residual toxicity, environmental pollution and microbial resistance (Rial-Otero *et al.,* 2005). Use of natural plants products is considered as an alternative to chemical fungicides for the control of phytopathogens (Parka *et al.,* 2002; Kanwal *et al.,* 2010; Riaz *et al.,* 2010a; Jabeen *et al.,* 2011). *Chenopodium album* is a very common weed of family Chenopodiaceae. Certain members of this family are known to exhibit antifungal potential (Kumar *et al.,* 2007; Javaid and Amin, 2009). Recently, Rauf and Javaid (2013) reported that root extract of *C. album* showed potent antifungal activity against *F. oxysporum* f. sp. *cepae.* The present study was, therefore, carried out to investigate the potential of soil incorporation of dry leaves of *C.album* for the control of basal rot disease of onion.

Materials and Methods

Isolation and Identification of Fungal Pathogen

Onion bulbs infected with basal rot disease were collected from a field of district Mandi Bahaud-Din of province Punjab, Pakistan in 2009. Infected tissues of onion bulb were surface-sterilized for 1 min using 1% solution of

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sodium hypochlorite and washed thoroughly in sterilized distilled water. Two growth media viz. malt extract agar (MEA) and potato dextrose agar (PDA) were used for fungal isolation. Pieces of the surface sterilized onion tissues were plated on the two growth media in 90-mm diameter Petri plates and incubated at 23ºC in the dark for 7 days. The isolated fungus was sub-cultured on PDA and MEA using a single spore technique for culture purification (Leslie and Summerell, 2006). Pure culture of the fungus was stored at 4°C in a refrigerator. The isolated fungal species was identified as *F. oxysporum* f. sp. *cepae* (Leslie and Summerell, 2006).

Pot Trials

In vivo antifungal bioassay against *F. oxysporum* f. sp. *cepae* was carried out by incorporating the powdered dry leaf biomass of *C. album*. Protocol given by Riaz *et al.* (2010b) was generally followed with some modification. For preparation of fungal inoculum, 1.0 kg boiled chickpea seeds were autoclaved at 121ºC for 30 min in transparent polypropylene bags. After cooling at room temperature, autoclaved chickpea seeds were inoculated with 14 days old culture of *F. oxysporum.* f. sp. *cepae* and incubated for 14 days at room temperature.

Plastic pots of diameter 6-cm and height 10-cm were filled with sandy loam texture soil at 350 g pot^{-1}. Pot soil was made sick by thorough mixing of 5 g pot^{-1} of *F. oxysporum.* f. sp. *cepae* inoculum prepared on chickpea. Pots were watered and left for one week for the establishment of fungal inoculum. After one week, pot soil was amended with 1, 2 and 3% (w/w) dried powdered leaf material of *C. album.* Pots of positive control were prepared by only inoculating them with the fungus while negative control treatment was without fungal inoculum as well as dry leaf amendment. Each treatment was replicated three times with 10 pots in each replicate. Pots were irrigated with tap water and left for 15 days.

Onion set bulbs of uniform size (2 cm diameter) of var. Phulkara, susceptible to basal rot disease were surface sterilized with 1% solution of sodium hypochlorite and thoroughly washed with sterilized water. Surface sterilized onion set bulbs were sown in each pot at 1 bulb per pot. After 3 days of sowing, pots were transferred to a growth chamber that was maintained at 18±2ºC and 10 h light period daily. Pots were irrigated with tap water whenever required. Data regarding germination, shoot and root growth, disease incidence and plant mortality were recorded after 10 weeks of seed sowing.

The data regarding disease incidence and mortality were recorded by applying the following formulae:

$$\text{Disease incidence } (\%) = \frac{\text{No. of diseased plants}}{\text{Total number of plants}} \times 100$$

$$\text{Mortality } (\%) = \frac{\text{No. of plants died due to disease}}{\text{Total number of plants}} \times 100$$

Laboratory Bioassays

Dry and powdered leaves of *C. album* (2.5 kg) were extracted with 7 L methanol twice for two weeks. Following filtration, the extract was evaporated on a rotary evaporator at 45ºC under vacuum to obtain crude methanolic extract. The crude methanolic extract was mixed in 500 mL distilled water. This mixture was thoroughly mixed with 500 mL of *n*-hexane and left for complete separation of aqueous and *n*-hexane layers in a separating funnel. The *n*-hexane phase was separated and the process was repeated till all the *n*-hexane soluble compounds were separated from the aqueous phase. The isolated *n*-hexane phase was evaporated under vacuum to yield 6.4 g of this fraction. The aqueous phase was further subjected to successive partitioning using 500 mL of each of chloroform, ethyl acetate and *n*-butanol to yield 2.7 g chloroform fraction, 3.2 g ethyl acetate fraction, and 3.8 g *n*-butanol fraction. Finally, the aqueous fraction was evaporated to dryness under reduced pressure to give 1.9 g gummy mass.

Antifungal activity of different fractions of methanolic leaf extract was evaluated against the target fungal species following Iqbal and Javaid (2012). Measured quantity (1.2 g) of different fractions of methanolic leaf extract was dissolved in 1 mL of dimethyl sulphoxide (DMSO) followed by addition of 5 mL of malt extract broth to prepare a solution of 200 mg mL^{-1}. Appropriate quantities of this stock solution and malt extracts were mixed to prepare 6 milliliters of each of the lower concentrations of 100, 50, …, 3.125 mg mL^{-1}. Various control treatments were similarly prepared by dissolving 1 mL of DMSO in 5 mL malt extract broth followed by serial double dilution. There was a control treatment corresponding to each extracts concentration both containing the same quantity of DMSO. Bioassays were conducted in 10 mL volume glass test tubes each containing 1 mL of medium. Test tubes were inoculated with one drop of spores of *F. oxysporum* f.sp. *cepae* aseptically. Each treatment was replicated three times. Test tubes were incubated for 7 days at room temperature. Thereafter, fungal biomass in each test tube was filtered, dried at 60°C and weighed.

Chloroform fraction of methanolic leaf extract exhibited the best antifungal activity and thus selected for further experimentation. Thin layer chromatography (TLC) was done for separation of different compounds present in these fractions. Isolation and purification was done by performing preparative thin layer chromatography (PTLC). The chloroform fraction of methanolic leaf extract chromatographed over TLC plates gave two compounds viz. A and B having R_f values of 0.17 and 0.44, respectively, by using solvent system of $CHCl_3$:MeOH (9:1).

The minimum inhibitory concentration (MIC) values of the compounds isolated from chloroform fraction and a reference synthetic fungicide mancozeb (80% WP, KSS) were tested in culture tubes by serial micro dilution assay (Masoko *et al.,* 2007; Jabeen *et al.,* 2011) with small

modifications. Eight milligrams of each of the two isolated compounds and mancozeb were dissolved in 0.5 mL DMSO and 1.5 mL malt extract was added to prepare 4000 µg mL^{-1} concentration. Medium was serially double diluted with malt extract broth in culture tubes to prepare 2000, 1000, …, 7.81 µg mL^{-1} concentrations. Control treatments were prepared by adding 0.5 mL DMSO in 1.5 mL ME broth and double diluted serially to prepare corresponding control for each dilution. Seven days old fungal culture was added to double distilled water to make spore suspension. One drop of the spore suspension was added to different concentrations of the growth medium in 5 mL volume glass tubes containing 1 mL of medium each. The culture tubes were incubated at room temperature and MIC was recorded after 24, 48 and 72 h (Javaid and Munir, 2012).

Statistical Analysis

All the data were analyzed by analysis of variance (ANOVA) followed by Duncan's Multiple Range Test using computer software SPSS.

Results

Pot Trials

Data presented in Fig. 1 reveals that there was 100% germination in negative control treatment. *F. oxysporum* f. sp. *cepae* inoculation significantly suppressed germination by 73%. There was a gradual increase in germination percentage as the leaf biomass dose was increased from 1 to 3%. The effect was significant as compared to positive control treatment where *F. oxysporum* f. sp. *cepae* inoculation was done without incorporation of leaf biomass. There was 63%, 90% and 100% germination in 1, 2 and 3% leaf biomass treatments, respectively.

Plants in non-inoculated negative control treatment did not show any disease symptoms either on aerial or underground plant parts In contrast, in positive control treatment, all plants were diseased due to *F. oxysporum* f. sp. *cepae* inoculation showing 100% disease incidence and 92% mortality. In general, all the concentrations of *C. album* dry leaf biomass managed the basal plate rot disease of onion to variable extents. The most pronounced antifungal effect was recorded in 3% dry leaf biomass amendment treatment, where a significant reduction of 73% and 89% was recorded in disease incidence and plant mortality, respectively. Lower doses of 1% and 2% dry leaf biomass found to be less effective resulting in significant 9 and 41% suppression in disease incidence and 40 and 59% decline in plant mortality, respectively (Fig. 2).

Inoculation of *F. oxysporum* f. sp. *cepae* significantly decreased the number and length of leaf as well as fresh and dry biomass of shoot in onion. Incorporation of different doses of dry leaf biomass alleviated the biotic stress of *F. oxysporum* f. sp. *cepae* and enhanced the shoot growth in onion to variable extents as compared to positive control.In

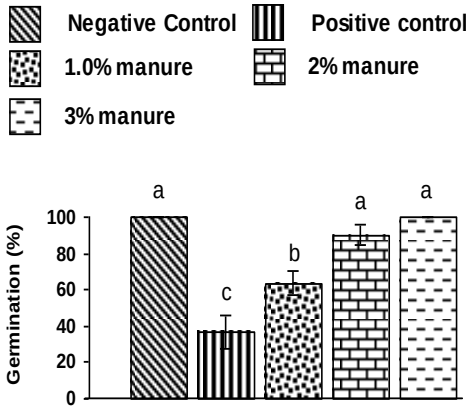

Fig. 1: Effect of *Fusarium oxysporum* f.sp. *cepa* inoculation and different doses of dry leaf biomass of *Chenopodium album* on germination of onion bulb. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Duncan's Multiple Range Test.
Negative control: No fungal inoculation or soil amendment
Positive control: *F. oxysporum* f.sp. *cepa* inoculation
1%, 2% and 3% leaf biomass: *F. oxysporum* f.sp. *cepa* inoculation + leaf biomass

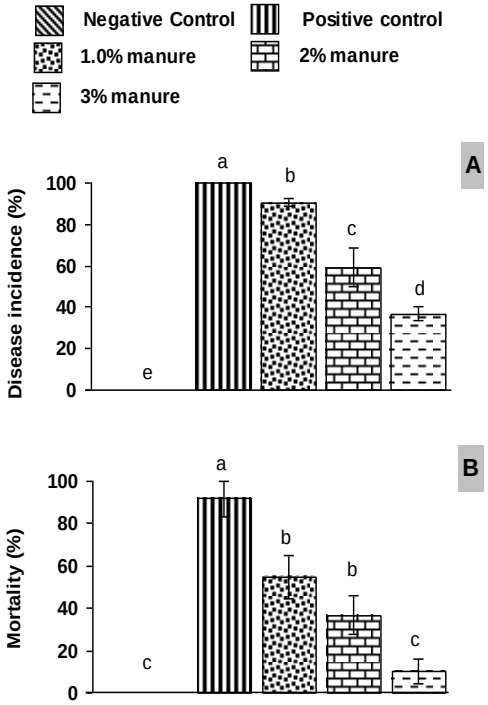

Fig. 2: Effect of *Fusarium oxysporum* f.sp. *cepa* inoculation and different doses of dry leaf manure of *Chenopodium album* on disease incidence and mortality of onion. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Duncan's Multiple Range Test
Negative control: No fungal inoculation or soil amendment
Positive control: *F. oxysporum* f.sp. *cepa* inoculation
1%, 2% and 3% leaf biomass: *F. oxysporum* f.sp. *cepa* inoculation + leaf biomass

general, increasing the leaf biomass dose resulted in a parallel increase in various shoot growth parameters. The effect of 2% and 3% dose was significant as compared to positive control in all the studied shoot growth parameters (Fig. 3).

Root growth was severely arrested due to *F. oxysporum* f. sp. *cepae* inoculation in positive control treatment. Roots became completely rotted due to fungal infection in this treatment. Incorporation of different doses of dry leaf biomass of *C. album* alleviated the biotic stress of *F. oxysporum* f. sp. *cepae* to variable extents. Generally, the effect of lower doses of 1 and 2% was insignificant, while 3% leaf biomass treatment significantly enhanced various root growth parameters as compared to positive control (Fig. 4).

Laboratory Bioassays

The effect of various fractions of methanolic extract on fungal growth is shown in Table 1. DMSO used to dissolve different fractions of methanolic leaf extract also exhibited some antifungal activity. Fungal growth appeared in all the concentrations of DMSO in control treatments. There was a parallel decrease in fungal biomass as the concentration of DMSO was increased from 0.0025 to 0.1666 mL mL^{-1} of the medium in these control treatment. Different organic solvent extractions exhibited variable antifungal activity. Chloroform fraction showed the maximum antifungal activity where 12.5–200 mg mL^{-1} concentrations of the extract completely inhibited the fungal growth. The effect of the lowest concentration of 3.125 mg mL^{-1} was also significant, where 96% reduction in fungal biomass was recorded over corresponding control treatment. Similarly, ethyl acetate and *n*-hexane fractions also found highly antifungal where 25–200 mg mL^{-1} and 50–200 mg mL^{-1} concentrations of the two extracts, respectively completely inhibited the fungal growth. *n*-Butanol fraction was found comparatively less effective where only 100 and 200 mg mL^{-1} concentrations completely inhibited the fungal growth while the effect of lower concentrations of 3.125–50 mg mL^{-1} was insignificant. Aqueous fraction of methanolic leaf extract generally stimulated the fungal growth.

Data regarding the antifungal activity of isolated compounds is presented in Table 2. Fungal growth appeared in control after 24 h incubation. In contrast, there was not any fungal growth in any of the mancozeb concentrations even after 72 h of incubation. The two isolated compounds showed different MIC values against target fungal species. TLC fraction A exhibited higher antifungal activity with MIC value 62.5 µg mL^{-1} than TLC fraction B with MIC value 250 µg mL^{-1} after 24 h of incubation. After 48 and 72 h incubation, the MIC values of TLC fractions A and B were 125 and 250 µg mL^{-1} (Table 2).

Discussion

F. oxysporum f. sp. *cepae* inoculated plants showed 100% disease incidence and 92% mortality. Soil amendment with *C. album* dry leaf biomass managed the basal plate rot disease of onion to variable extents. The maximum reduction of 73% and 89% was recorded in disease incidence and plant mortality, respectively due to 3% dry *C. album* leaf amendment. Likewise, 1% and 2% dry leaf biomass amendment also reduced disease incidence and plant mortality but to a lower extent. Earlier, Riaz *et al.* (2010b) reported that soil amendment with *C. album* leaves significantly reduced incidence of corm rot disease of gladiolus (*Gladiolus grandiflorus* cv. Aarti) caused by *F.*

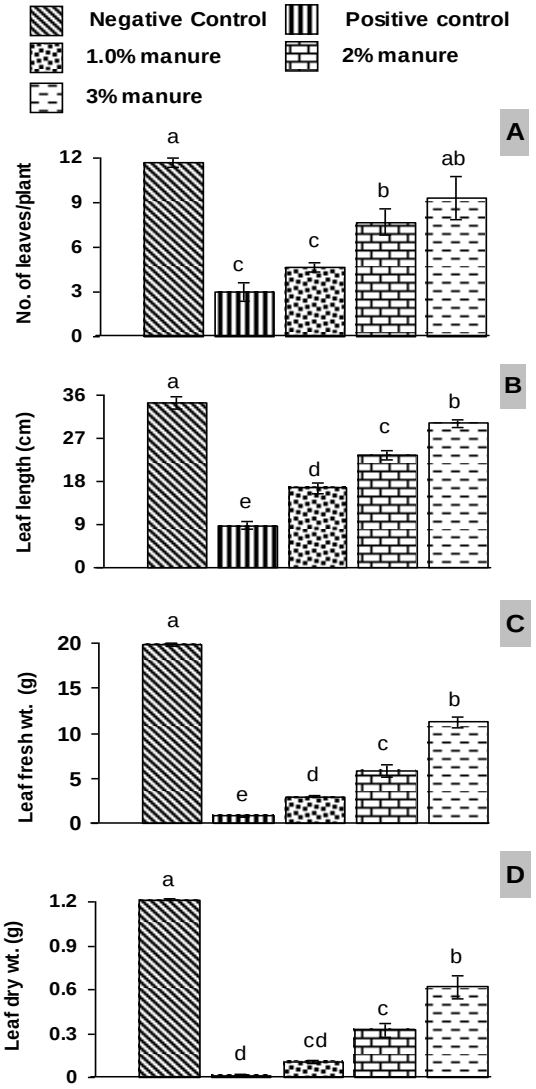

Fig. 3: Effect of *Fusarium oxysporum* f.sp. *cepa* inoculation and different doses of dry leaf manure of *Chenopodium album* on leaf growth of onion. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Duncan's Multiple Range Test
Negative control: No fungal inoculation or soil amendment
Positive control: *F. oxysporum* f.sp. *cepa* inoculation. 1%, 2% and 3% leaf biomass: *F. oxysporum* f.sp. *cepa* inoculation + leaf biomass

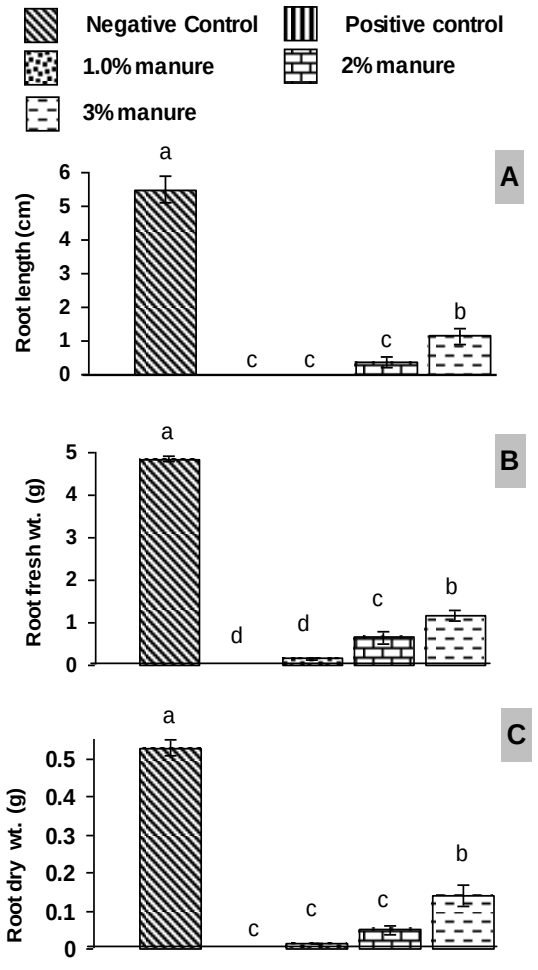

Fig. 4: Effect of *Fusarium oxysporum* f.sp. *cepa* inoculation and different doses of dry leaf manure of *Chenopodium album* on root growth of onion. Vertical bars show standard errors of means of three replicates. Values with different letters at their top show significant difference ($P \leq 0.05$) as determined by Duncan's Multiple Range Test
Negative control: No fungal inoculation or soil amendment
Positive control: *F. oxysporum* f.sp. *cepa* inoculation
1%, 2% and 3% manure: *F. oxysporum* f.sp. *cepa* inoculation + leaf manure

oxysporum f. sp. *gladioli.* It is likely that antifungal compounds such as trans-2, transe-4 hexadienedial (Tahara *et al.*, 1994), saponins (Lavaud *et al.*, 2000) and phenolics (Laghari *et al.*, 2011) released from decomposing *C. album* leaf biomass into the pot soil, suppressed growth of *F. oxysporum* f. sp. *cepae* and consequently reduced disease incidence and plant mortality.

In laboratory bioassays, a series of control treatment were designed, which corresponded to different concentrations of DMSO in various extract treatments. In the present study, higher concentrations of DMSO also adversely affected fungal growth. Earlier studies have shown variable effects of DMSO on fungal growth that generally varied with the fungal species (Javaid and Samad, 2012; Rauf and Javaid, 2013). In laboratory bioassays, different fractions of methanolic extracts exhibited variable antifungal activities that could be attributed to different polarity nature of the organic solvents used for partitioning of methanolic extract. These organic solvents ranged from non-polar *n*-hexane to highly polar *n*-butanol. Depending upon their polarity nature, different compounds in methanolic extract were dissolved in different organic solvents. Comparable antifungal activity of various organic solvent fractions of methanolic extracts of *Datura metel, WIthania somnifera, Syzygium cumini* and *Coronopus didymus* has also been reported against other fungal pathogens such as *Sclerotium rolfsii*, *Alternaria alternata*,

Table 1: Effect of different concentrations of *n*-hexane, chloroform, ethyl acetate, *n*-butanol and aqueous fraction of methanolic leaf extract of *Chenopodium album*on *in vitro* growth of *Fusarium oxysporum* f. sp. *cepae*

Methanolic fraction	Conc. of DMSO (mL mL^{-1})	Extract conc. (mg mL^{-1})	Fungal biomass (mg)
Control	0.1666	0	0.33 lm
	0.0833	0	1.5 i
	0.0416	0	1.7 i
	0.0208	0	1.76 hi
	0.0104	0	2.26 e-g
	0.0052	0	2.4 d-f
	0.0025	0	2.73 bc
n-hexane	0.1666	200	0 m
	0.0833	100	0 m
	0.0416	50	0 m
	0.0208	25	1.1 j
	0.0104	12.5	1.46 i
	0.0052	6.25	2.3 d-g
	0.0025	3.125	2.6 b-d
Chloroform	0.1666	200	0 m
	0.0833	100	0 m
	0.0416	50	0 m
	0.0208	25	0 m
	0.0104	12.5	0 m
	0.0052	6.25	0.03 m
	0.0025	3.125	0.1 m
Ethyl acetate	0.1666	200	0 m
	0.0833	100	0 m
	0.0416	50	0 m
	0.0208	25	0 m
	0.0104	12.5	0.06 m
	0.0052	6.25	0.33 lm
	0.0025	3.125	0.43 l
n-butanol	0.1666	200	0 m
	0.0833	100	0 m
	0.0416	50	1.33 i
	0.0208	25	1.7 i
	0.0104	12.5	2.03 gh
	0.0052	6.25	2.16 f-g
	0.0025	3.125	2.5 c-e
Aqueous	0.1666	200	0.16 lm
	0.0833	100	0.73 k
	0.0416	50	1.76 hi
	0.0208	25	2.3 bc
	0.0104	12.5	2.86 ab
	0.0052	6.25	3.06 a
	0.0025	3.125	3.16 a

In vertical column, values with different letters at their top show significant difference ($p \leq 0.05$) as determined by Duncan's Multiple Range Test

Table 2: MIC values of isolated compounds from *Chenopodium album* and synthetic fungicide Mancozeb against *Fusarium oxysporum* f. sp. *cepae* after 24, 48 and 72 h

Treatments	Concentrations µg mL^{-1}								
	2000	1000	500	250	125	62.5	31.25	15.62	7.81
After 24 h incubation period									
*Control	+	+	+	+	+	+	+	+	+
TLC fraction A	-	-	-	-	-	-	+	+	+
TLC fraction B	-	-	-	-	+	+	+	+	+
Mancozeb	-	-	-	-	-	-	-	-	-
After 48 h incubation period									
TLC fraction A	-	-	-	-	-	+	+	+	+
TLC fraction B	-	-	-	-	+	+	+	+	+
Mancozeb	-	-	-	-	-	-	-	-	-
After 72 h incubation period									
TLC fraction A	-	-	-	-	-	+	+	+	+
TLC fraction B	-	-	-	-	+	+	+	+	+
Mancozeb	-	-	-	-	-	-	-	-	-

\+ Fungal growth appear

– No fungal growth

*__Note:__ Concentrations are only for compounds A and B and mancozeb

Ascochyta rabiei and *Macrophomina phaseolina* (Iqbal and Javaid, 2012; Javaid and Munir, 2012; Javaid and Saddique, 2012; Javaid and Samad, 2012).

In laboratory bioassays, chloroform fraction of methanolic leaf extract showed the best antifungal activity. Two unidentified compounds were separated from this fraction. Compound A (R_f 0.17) exhibited the pronounced antifungal activity with respect to the activity of synthetic fungicide mencozeb. Earlier, Tahara *et al.* (1994) reported a metabolic mucondialdehyde (trans-2, transe-4 hexadienedial) from the leaves of *C. album* exhibiting pronounced antifungal activity. Similarly, Lavaud *et al.* (2000) have identified three saponins namely calenduloside E, chikusetsaponin IV and 3-o-[3-o (2-o-glycolyll)-glycoxylyl β-D-glucuronopyranosyl] oleanolic acid, from leaves of *C. album*, which may be responsible for antifungal activity against *F. oxysporum* f.sp. *cepae* (Chapagain *et al.*, 2007). Laghari *et al.* (2011) isolated different phenolic acids from leaves of *C. album*, which could be responsible for antifungal activity against the test pathogenic fungal species (Báidez *et al.*, 2006).

Conclusion

The present study concludes that basal rot disease of onion can effectively be managed by soil amendment with dry leaves of *C. album* at 3% (w/w). The unidentified compound A from chloroform fraction of methanolic leaf extract of *C. album* is very effective. Further studies are required to identify this compound using various spectroscopic techniques.

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